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A C-terminal aldehyde analog of the insect kinins inhibits diuresis in the housefly

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ABSTRACT

The insect kinins are present in a wide variety of insects and function as potent diuretic peptides in flies. A C-terminal aldehyde insect kinin analog, Fmoc-RFFPWG-H (R-LK-CHO), demonstrates stimulation of Malpighian tubule fluid secretion in crickets, but shows inhibition of both *in vitro* and *in vivo* diuresis in the housefly. R-LK-CHO reduced the total amount of urine voided over 3 h from flies injected with 1 μL of distilled water by almost 50%. The analog not only inhibits stimulation of housefly fluid secretion by the native kinin Musdo-K, but also by thapsigargin, a SERCA inhibitor, and by ionomycin, a calcium ionophore. The activity of R-LK-CHO is selective, however, as related C-terminal aldehyde analogs do not demonstrate an inhibitory response on housefly fluid secretion. The selective inhibitory activity of R-LK-CHO on housefly tubules represents an important lead in the development of environmentally friendly insect management agents based on the insect kinins.

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1. Introduction

The insect kinins share a highly conserved C-terminal pentapeptide sequence Phe-Xaa-Xbb-Trp-Gly-NH$_2$, where Xaa can be Tyr, His, Ser or Asn, and Xbb can be Ala but is generally Ser or Pro [8]. They have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera, and Orthoptera. The first members of this insect neuropeptide family were isolated on the basis of their ability to stimulate contractions of the isolated cockroach hindgut [7,9], but they are also potent diuretics that stimulate the secretion of primary urine by Malpighian (renal) tubules,

organs involved in the regulation of salt and water balance, and the excretion of toxic wastes [2]. In the migratory locust (*Locusta migratoria*) the insect kinins and the corticotropin releasing factor (CRF)-related peptide, which are co-localized in abdominal neurosecretory cells, act synergistically to stimulate Malpighian tubule fluid secretion [2,25]. In the housefly, muscakinin has been implicated in the control of diuresis in response to hypervolemia [4] and elicits a four- to five-fold increase in fluid secretion by isolated Malpighian tubules, more than twice the response observed with the larger CRF-related Musca-DP [2,25]. In addition, insect kinins, and/or analogs, have been reported to inhibit weight gain by

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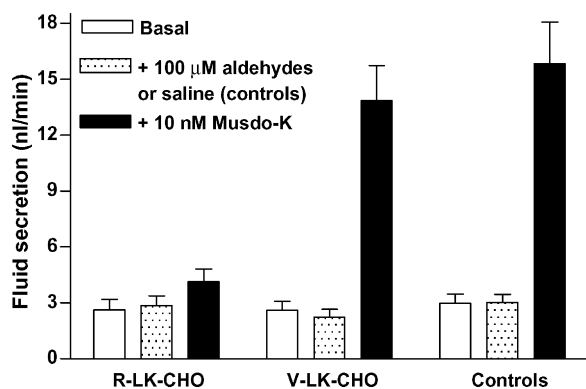| 16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT **Same as Report (SAR)**	18. NUMBER OF PAGES **7**	19a. NAME OF RESPONSIBLE PERSON
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Fig. 1 – The aldehyde analogs R-LK-CHO and V-LK-CHO have no effect on basal secretion by isolated Malpighian tubules, but the former prevents stimulation by the native kinin Musdo-K. Bars represent the means and vertical lines +1 S.E.M. for the rate of secretion by 5–7 tubules measured over 25 min periods. After determining the basal rate of secretion, tubules were challenged with 100 μM of the aldehyde analogs or saline (controls) alone, and then in combination with 10 nM Musdo-K.

larvae of the tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) [19,24], both serious agricultural pests.

Structurally, the insect kinins require an intact C-terminal pentapeptide sequence for full cockroach myotropic and cricket diuretic activity, which therefore represents the active core [9]. An Ala-replacement analog series of the insect kinin active core region confirms the importance of the Phe and Trp side chains, because these are the only two replacements which lead to complete loss of myotropic and diuretic activity [15,17,22]. Analysis of conformationally restricted insect kinin analogs indicated that the active conformation featured a *cis* Pro in the third position of a type-VI β-turn over core residues 1–4, or Phe-Phe-**Pro**-Trp (Fig. 1) [11,14–18,20,22]. Activity in both myotropic and diuretic assays is almost completely lost when the C-terminal amide of the insect kinins is replaced with a negatively charged acid moiety. However, activity is retained in analogs that feature neutral group replacements, such as found in C-terminal ester, thioester and modified amide analogs [13]. In addition, a C-terminally modified insect kinin analog featuring an aldehyde in place of an amide group was reported to stimulate secretion by cricket Malpighian tubules [12]. The same analogs were also shown to enhance the inhibition of weight gain and to induce significant mortality in *H. zea* moth larvae [12].

Aldehydes can form reversible imine bonds with amino groups. Peptide analogs containing reversible binding moieties, such as an aldehyde, at the C-terminus have been reported to inhibit various classes of proteolytic enzymes [1,6,23], and have been shown to both enhance and modify the activity of insect kinins in a developmental assay in larval moths [12]. Here we report on the evaluation of insect kinin analogs containing a C-terminal aldehyde in both *in vitro* and *in vivo* diuretic assays of the housefly *Musca domestica*.

2. Materials and methods

2.1. Chemistry

The C-terminal aldehyde insect kinin analogs R-LK-CHO (Fmoc-Arg-Phe-Phe-Pro-Trp-Gly-H) and V-LK-CHO (Boc-Val-Phe-Phe-Pro-Trp-Gly-H) were synthesized as previously described [19,20]. Peptide aldehydes, Fmoc-Arg-Phe-Phe-Aib-Trp-Gly-H and Fmoc-Arg-Ala-His-Pro-Trp-Gly-H were synthesized by the solid-phase method on ABI 433A Peptide Synthesizer with modified FastMoc0.25 procedure (0.25 mM scale, prolonged double coupling), using the Fmoc-strategy and starting from Weinreb AM resin (Novabiochem, 0.63 mM/g). Fmoc protecting groups were removed by 20% piperidine in NMP. A four-fold excess of the respective Fmoc-amino acid was preactivated using HBTU (1 equiv.)/HOBt (1 equiv.) in NMP and coupling reactions were base catalyzed with DIPEA (2 equiv.). Amino acid side chain protecting groups were Pbf for Arg, Trt for His and Boc for Trp. Completed peptides on the resin were transferred to the plastic syringe, washed with DCM (3$\times$) and MeOH (3$\times$) and dried on air. Side-chain deprotection was performed by treatment with TFA:H$_2$O:TIS (95:2.5:2.5, v/v/v, 10 mL/g peptidyl-resin) 2$\times$ 1 h. After washing 3$\times$ DCM and 3$\times$ MeOH, resin was dried *in vacuo* over P$_2$O$_5$ overnight. Then peptidyl-resins were suspended in dry THF (3 mL) in 25 mL glass flask under argon, swelled with gentle stirring for 1 h, then cooled down in ice-water bath for 30 min. Cleavage of the peptide aldehydes from the resin was performed by LiAlH$_4$ (Aldrich, 1 M solution in THF, 2 molar equivalents plus 1 equiv. for each acidic H atom (addition of drops from the syringe through rubber septum) for 30 min at 0 °C with stirring. The reaction was quenched with careful addition of 1 M KH$_2$PO$_4$ and stirred until has reached room temperature. Resulting suspension was filtered, washed with water 2$\times$ 10 mL, filtrate combined with washings and desalted using Waters C$_{18}$ Sep Pak cartridge. Residual solid on the funnel was washed 3$\times$ 10 mL 80% acetonitrile in water containing 0.1% TFA and combined extracts were freeze-dried on a Savant SC110 SpeedVac, and then desalted as above.

The C-terminal aldehyde analogs were purified on a Delta-Pak C$_{18}$ reverse-phase column (8 mm $\times$ 100 mm, 15 μm particle size, 100 A pore size), and on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 mL/min. Delta-Pak C-18 retention times: Fmoc-Arg-Phe-Phe-Aib-Trp-Gly-H, t_R = 25.5 min; Fmoc-Arg-Ala-His-Pro-Trp-Gly-H, t_R = 19.5 min. Amino acid analysis was carried out under previously reported conditions [13] and used to quantify the peptides and to confirm identity, leading to the following analysis: Fmoc-Arg-Phe-Phe-Aib-Trp-Gly-H, F[2.0], R[0.7]; Fmoc-Arg-Ala-His-Pro-Trp-Gly-H, A[1.0], H[0.9], P[1.0], R[1.0]. The identities of the peptide analogs were confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical Ltd., Manchester, UK) with the presence of the following

molecular ions: Fmoc-Arg-Phe-Phe-Aib-Trp-Gly-H, , 1001.7 [MH$^+$]; Fmoc-Arg-Ala-His-Pro-Trp-Gly-H, 945.9 [M + Na$^+$].

2.2. Malpighian tubule fluid secretion assay

The diuretic assay has been described in detail elsewhere [2]. In brief, the posterior directed pair of Malpighian tubules were removed from adult female houseflies 3–4 days old and were transferred to 10 μL drops of bathing fluid beneath water-saturated liquid paraffin in a Sylgard-lined Petri dish. The bathing fluid used in this study was a 1:1 mixture of Schneider's *Drosophila* medium and housefly saline with the following composition (in mM/L): NaCl, 135; KCl, 20; CaCl$_2$, 2; MgCl$_2$, 8.5; NaHCO$_3$, 10.2; NaH$_2$PO$_4$, 4.3; HEPES, 15; glucose, 20; pH adjusted to 7.0 with 1 M NaOH. Urine escapes from the proximal end of the tubule and collects as a discrete droplet in the paraffin oil. Droplets of secreted urine were collected at intervals and their diameter measured as they rested on the non-wettable Sylgard base. Urine volumes were calculated assuming the drops to be perfect spheres and the rate of excretion determined by dividing the volume by the time over which urine was collected. Tubules were allowed to equilibrate for 60 min after which the bathing fluid was replaced and the secreted urine removed. Urine samples were then collected over measured time intervals before and after challenging tubules with test compounds.

2.3. Measurement of diuresis in intact flies

Water loss from adult male flies 3–4 days post-emergence was measured using a Sable Systems (Henderson, NV, USA) flow-through humidity analyser (RH-100) as described previously [3]. Episodes of urine excretion are readily seen as sporadic peaks of heightened water loss on digitized recordings (DATACAN V, Sable Systems) from the humidity analyzer. The volume of urine excreted is obtained by integrating the area beneath the spike-like peaks using DATACAN V [3]. Recordings lasted for 3 h and urine losses were summed over consecutive 15 min periods.

Diuresis can be induced by intrahemocoelic injections of saline or distilled water. These injections were performed with a UMP-1 ultramicropump (WPI, Sarasota, FL, USA) fitted with a 50 μL Hamilton syringe and a needle manufactured from a glass micropipette (tip diameter 50–60 μm) as described by Coast [3]. All experiments were performed at room temperature, which varied between 23 and 25 °C during the course of these studies.
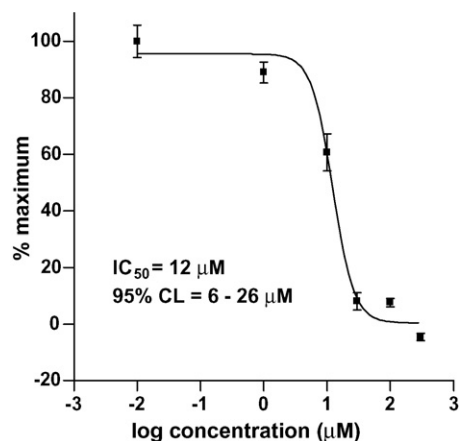

Fig. 2 – Dose–response curve for R-LK-CHO showing the percentage inhibition of the diuretic activity of 10 nM Musdo-K. Data points are the means of five to seven determinations and vertical lines ±1 S.E.M.

3. Results

The *in vitro* diuretic activity of the C-terminal aldehyde insect kinin analogs **Fmoc-Arg**-Phe-Phe-Pro-Trp-Gly-H (R-LK-CHO) and **Boc-Val**-Phe-Phe-Pro-Trp-Gly-H (V-LK-CHO) has been previously reported for cricket Malpighian tubules [12] and is summarized in Table 1. The EC$_{50}$ values for these two analogs are 250 and 30 nM, respectively; considerably less potent than the C-terminally amidated hexapeptide insect kinin Arg-Phe-Phe-Pro-Trp-Gly-NH$_2$ (EC$_{50}$ = 0.02 nM). Although the potencies of R-LK-CHO and V-LK-CHO in the cricket fluid secretion assay are about four-orders of magnitude less, they are capable of matching the maximal response of the native insect kinins.

By contrast, in the diuretic assay using housefly tubules, neither V-LK-CHO nor R-LK-CHO demonstrates any agonist response when tested at 100 μM (Fig. 1; Table 2). However, when the same tubules were challenged with 10 nM Musdo-K, 100 μM R-LK-CHO completely blocked the stimulation of fluid secretion by this native kinin neuropeptide, whereas 100 μM V-LK-CHO was without effect (Fig. 1). The IC$_{50}$ for the inhibition of Musdo-K diuretic activity by R-LK-CHO is 12 μM (Fig. 2). In marked contrast, the R-LK-CHO analog Fmoc-Arg-**Ala-His**-Pro-Trp-Gly-H ([Ala^2,His^3]R-LK-CHO) demonstrates diuretic activity in the housefly Malpighian

Table 1 – *In vitro* diuretic activity of C-terminal insect kinin analogs in the cricket *Acheta domesticus*	
Insect kinin analog	Stimulation of Malpighian tubule fluid secretion—EC$_{50}$ (10^{-9} M) (% maximal response)
Arg-Phe-Phe-Pro-Trp-Gly-NH$_2$	0.02 (100) [12]
Fmoc-Arg-Phe-Phe-Pro-Trp-Gly-H (R-LK-CHO)	250 (100) [12]
Boc-Val-Phe-Phe-Pro-Trp-Gly-H (V-LK-CHO)	30 (100) [12]

| Table 2 – *In vitro* diuretic activity of C-terminal insect kinin analogs in the housefly *Musca domestica*	
Insect kinin analog	Stimulation of Malpighian tubule fluid secretion— EC$_{50}$ (10^{-6} M) [95%CL] (% maximal response)
Boc-Val-Phe-Phe-Pro-Trp-Gly-H	Inactive
Fmoc-Arg-Phe-Phe-Pro-Trp-Gly-H	Inactive (inhibitory)
Fmoc-Arg-**Ala-His**-Pro-Trp-Gly-H	2.29 [2.24–2.34] (100)
Fmoc-Arg-Phe-Phe-**Aib**-Trp-Gly-H	Inactive
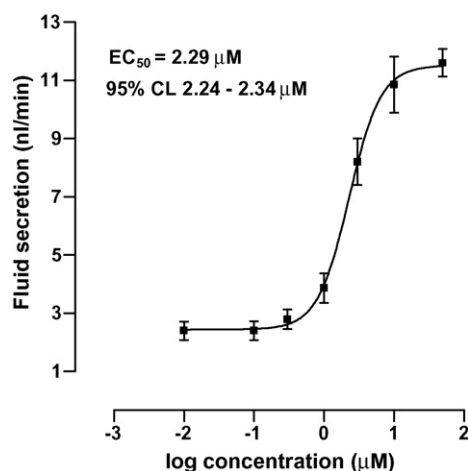
Fig. 3 – Dose–response curve for the diuretic activity of the aldehyde analog Fmoc-RAHPWG-H. Data points are the means of five to seven determinations and vertical lines ±1 S.E.M.

tubule fluid secretion assay with an EC_{50} of 2 μM (Table 2; Fig. 3). The R-LK-CHO analog Fmoc-Arg-Phe-Phe-**Aib**-Trp-Gly-H ([Aib^4]R-LK-CHO) is inactive in the *in vitro* housefly diuretic assay and fails to inhibit the stimulatory response of Musdo-K (Table 2; Fig. 4). The residue α-aminoisobutyric acid (Aib) had been previously inserted in replacement of the Pro in the core region of insect kinin analogs with significant retention of activity observed in *in vitro* and *in vivo* cricket and housefly diuretic assays [19].

Previously, injection of 50 pmol Musdo-K dissolved in 1 μL of housefly saline was shown to stimulate urine output *in vivo* [3]. Co-injection of 50 pmol R-LK-CHO and 50 pmol Musdo-K significantly reduced the *in vivo* diuretic activity of the native kinin (Fig. 5). This is evident from the marked reduction in urine output over the first 45 min post-injection, which in flies injected with Musdo-K alone was 191 ± 37 nL (N = 6; from [3])
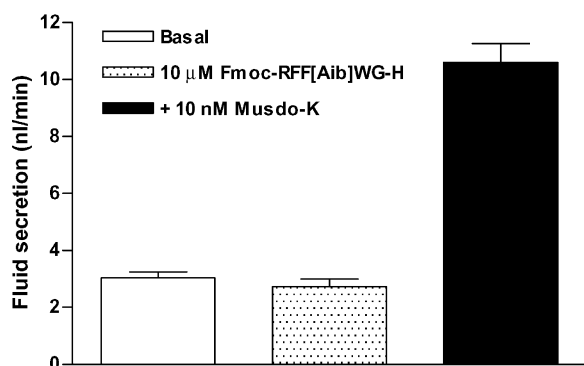

Fig. 4 – The aldehyde analog Fmoc-RFF[Aib]WG-H has no effect on basal secretion and does not prevent stimulation by the native kinin Musdo-K. Bars represent the means and vertical lines +1 S.E.M. for the rate of secretion by eight tubules measured over 25 min periods. After determining the basal rate of secretion, tubules were challenged with 10 μM of the aldehyde analog alone and then in combination with 10 nM Musdo-K.
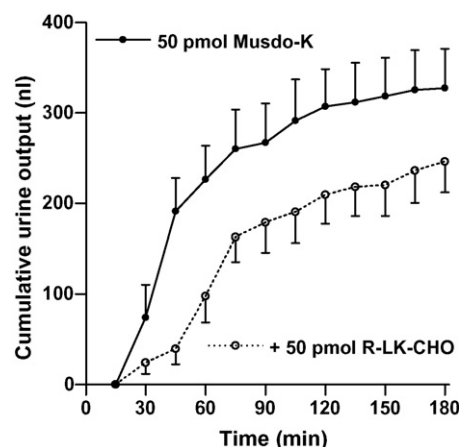

Fig. 5 – R-LK-CHO attenuates the *in vivo* diuretic activity of Musdo-K. The volume of urine excreted by individual flies was measured over 15 min periods for 3 h in insects injected with 1 μL saline containing either 50 pmol Musdo-K alone (solid circles, solid line) or in combination with 50 pmol of the aldehyde analog (open circles, dotted line). Data points show the means ± 1 S.E.M of the cumulative urine output in six (Musdo-K alone) and nine (+R-LK-CHO) flies.

compared with just 39 ± 17 nL (N = 9) in flies injected with Musdo-K plus R-LK-CHO ($P < 0.001$, paired t-test). The latter value is not significantly different ($P = 0.211$; unpaired t-test) from the cumulative urine loss at 45 min post-injection in control flies injected with 1 μL saline alone (80 ± 25; N = 10; from [3]). Thereafter, flies injected with Musdo-K alone or plus R-LK-CHO excrete urine at similar rates as evidenced by the curves for cumulative urine output running parallel to one another (Fig. 5).

Diuresis by intact flies is also stimulated by abdominal distension resulting from the intrahemocoelic injection of saline [3]. To investigate the effect of R-LK-CHO on this hypervolemic response we measured urine output in flies injected with 3 μL housefly saline with or without the addition of either 50 or 250 pmol of the aldehyde analog (Fig. 6). Over 3 h, flies injected with 3 μL saline alone voided 217 ± 26 nL (N = 10), but this was reduced to 158 ± 25 nL (N = 6) by the addition of 50 pmol R-LK-CHO to the saline, although the difference is not significant ($P = 0.150$). Increasing the amount of R-LK-CHO to 250 pmol further reduced urine output to 130 ± 19 nL (N = 6), which is significantly less ($P < 0.05$) than for the injection of saline alone.

An effective way of stimulating diuresis *in vivo* is to inject flies with distilled water, a response that may be due in large part to the reduction in hemolymph osmotic concentration since it is considerably greater than the effect of injecting the same volume of saline [3]. To test the effect of the aldehyde analog on this diuresis, flies were injected with 1 μL distilled water with or without the addition of 50 pmol R-LK-CHO (Fig. 7). Flies injected with distilled water alone voided 494 ± 18 nL (N = 7) of urine over 3 h, which is significantly greater ($P < 0.01$; unpaired t-test with Welch correction) than for insects injected with 1 μL of distilled water containing 50 pmol R-LK-CHO.
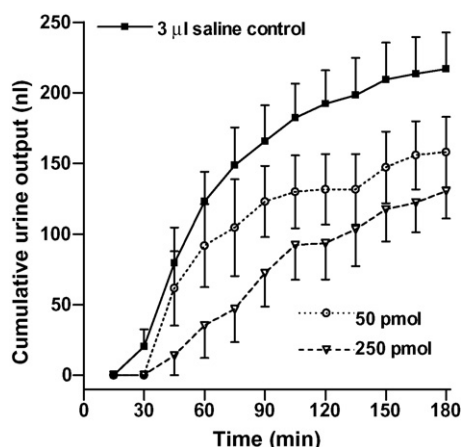
Fig. 6 – R-LK-CHO attenuates the *in vivo* diuretic response to hypervolemia induced by the injection of 3 μL of saline. The volume of urine excreted by individual flies was measured over 15 min periods for 3 h in insects injected with saline alone (solid squares, solid line) or saline containing either 50 pmol (open circles, dotted line) or 250 pmol (open triangle, dashed line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in 10 (saline alone) and 6 (+R-LK-CHO) flies.
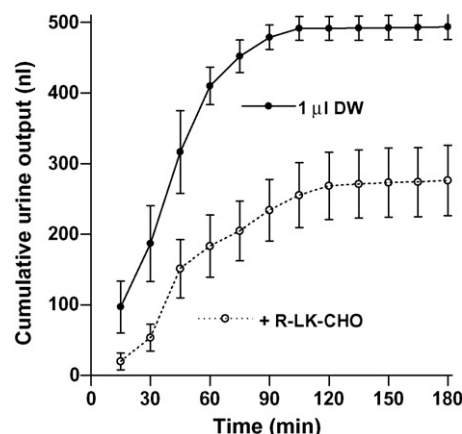

Fig. 7 – R-LK-CHO significantly reduces the amount of urine excreted by flies injected with 1 μL of distilled water. The volume of urine excreted by individual flies was measured over 15 min periods for 3 h in insects injected with distilled water alone (solid squares, solid line) or with distilled water containing 50 pmol (open circles, dotted line) of the aldehyde analog. Data points show the means ± 1 S.E.M of the cumulative urine output in seven (distilled water) and nine (+R-LK-CHO) flies.

The ability of R-LK-CHO to markedly reduce the diuresis evoked by the injection of distilled water suggests it is doing more than acting as a kinin antagonist. Evidence for this is presented in Fig. 8, which shows that R-LK-CHO inhibits not only the diuretic activity of Musdo-K, but also prevents stimulation of fluid secretion by thapsigargin, which elevates intracellular Ca^{2+} levels by blocking smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity. Fig. 8A shows the results of an experiment in which isolated Malpighian tubules were first challenged with 10 nM Musdo-K, which elicits a five-fold increase in fluid secretion. Following the addition of 30 μM R-LK-CHO to the same tubules there is an immediate reduction in fluid secretion, and they do not respond when challenged after 100 min with 20 μM thapsigargin. On the other hand, control tubules challenged with 20 μM thapsigargin after

105 min show a robust stimulation of fluid secretion (Fig. 8B), suggesting that the inhibitory effect of the aldehyde analog is a toxic one and not due to antagonism of the insect kinin receptor. In support of this, 100 μM of the aldehyde analog also prevents stimulation of fluid secretion by 10 μM ionomycin, which is a calcium ionophore that will promote Ca^{2+} entry into tubule cells from the bathing medium (Fig. 9).

4. Discussion

We had previously shown that insect kinin analogs in which the C-terminal amide was replaced by an aldehyde moiety stimulated fluid secretion by cricket Malpighian tubules and wished to determine whether they had diuretic activity *in vivo*.
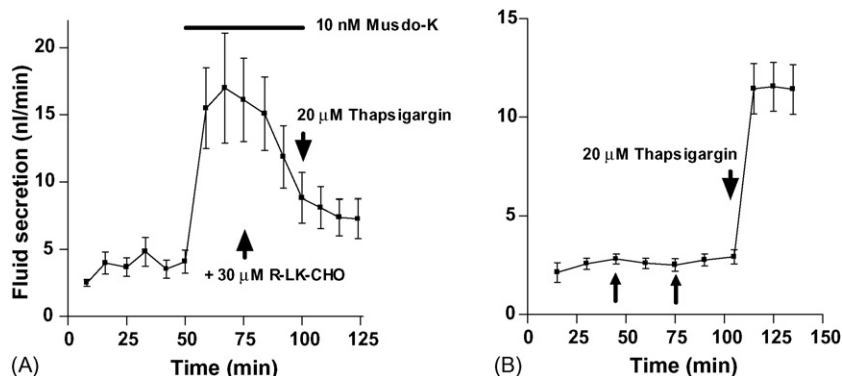

Fig. 8 – (A) The aldehyde analog R-LK-CHO (30 μM) reverses the increase in Malpighian tubule fluid secretion in response to 10 nM Musdo-K and prevents stimulation by 20 μM thapsigargin. (B) Addition of 20 μM thapsigargin to control tubules (saline changes indicated by small arrows) results in a robust stimulation of fluid secretion. Data points show mean values for the rate of secretion (±1 S.E.M) of individual tubules measured at 10–15 min intervals in six replicates.
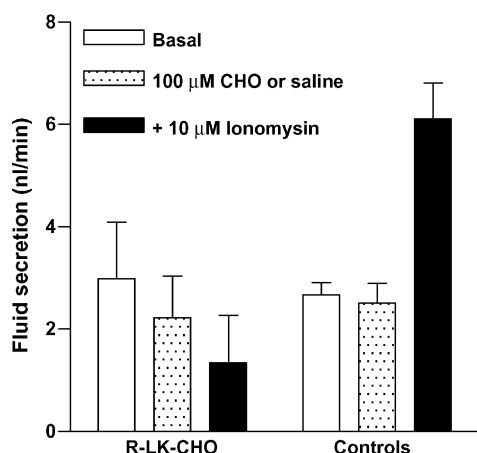
Fig. 9 – R-LK-CHO blocks the stimulation of fluid secretion by ionomycin. Bars represent the means and vertical lines +1 S.E.M. for the rate of secretion by five to six tubules measured over 25 min periods. After determining the basal rate of secretion, tubules were challenged with 100 μM of the aldehyde analog (or with saline; controls) alone and then in combination with 10 μM ionomycin.

Unfortunately, it has not been possible to develop a reliable *in vivo* assay for diuresis in crickets, but such assays are available for houseflies [3,4]. The aldehyde analogs R-LK-CHO and V-LK-CHO were initially screened at 100 μM for activity on housefly Malpighian tubules. The concentration used is ≥400-fold higher than the EC_{50} values for R-LK-CHO (0.25 μM) and V-LK-CHO on cricket tubules, but it is known that truncated analogs of Musdo-K have greatly reduced potency on housefly tubules [5]. Neither of the two analogs stimulated fluid secretion, but tubules exposed to R-LK-CHO did not respond when subsequently challenged with a supramaximal concentration (10 nM) of Musdo-K. In contrast, the same concentration of Musdo-K elicited a marked diuretic response in tubules that had first been exposed to V-LK-CHO. The inhibitory effect of R-LK-CHO on the diuretic activity of 10 nM Musdo-K was dose-dependent with an IC_{50} of 12 μM, which compares favorably with the EC_{50} of an N-terminal truncated Musdo-K analog of similar length (Musdo-K$_{[9–15]}$ EC_{50} ~1 μM; [5]).

Other aldehyde analogs were also tested on housefly tubules for diuretic activity and their ability to inhibit stimulation of fluid secretion by 10 nM Musdo-K. Of these, Fmoc-RAHPWG-H, which closely resembles R-LK-CHO, had diuretic activity with an EC_{50} of 2.3 μM. On the other hand, an Aib containing aldehyde analog, Fmoc-RFF[Aib]WG-H, had no effect on fluid secretion and, in common with V-LK-CHO, it did not inhibit the diuretic activity of 10 nM Musdo-K. The inhibitory activity of R-LK-CHO is thus highly specific and not a generalized effect of the aldehyde moiety.

The ability of R-LK-CHO to prevent stimulation of fluid secretion by Musdo-K was of considerable interest, because the analog might be used as a probe to investigate the role of the native kinin in the control of diuresis *in vivo*. By using a high sensitivity flow through humidity analyzer we were able to record the episodic excretion of urine from intact houseflies

[3]. R-LK-CHO inhibits the *in vivo* activity of Musdo-K as evidenced by a marked reduction in the amount of urine voided over the initial 45 min post-injection period in flies injected with 50 pmol each of the analog and the kinin compared with the kinin alone. R-LK-CHO was also shown to reduce the volume of urine voided during a diuresis initiated by the injection of 3 μL saline alone, although a larger dose (250 pmol) of the aldehyde was needed for a significant effect, probably because of the expanded hemolymph volume. The diuretic response to hypervolemia is partly attributable to the release of Musdo-K from neurohaemal sites into the circulation, and the inhibitory effect is consistent with a selective effect of R-LK-CHO at the kinin receptor.

Injection of 1 μL distilled water is a more effective stimulant of diuresis than is the injection of 3 μL of saline, with total urine loss over 3 h being more than double that in saline injected flies. This difference in part reflects an autonomous response of the Malpighian tubules, which secrete at higher rates when the osmotic concentration of the bathing fluid is reduced, as would occur after injecting flies with distilled water [4]. R-LK-CHO would not be expected to have any effect on the autonomous response of Malpighian tubules to haemolymph dilution, and yet it reduced the total amount of urine voided over 3 h from flies injected with 1 μL of distilled water by almost 50%.

The markedly reduced urine output from flies injected with 1 μL distilled water containing 50 pmol R-LK-CHO suggests this analog has a toxic effect on Malpighian tubules, and in support of this we have show that it blocks stimulation of fluid secretion by thapsigargin, a SERCA inhibitor, and by ionomycin, a calcium ionophore. Kinin neuropeptides use Ca^{2+} as a second messenger to open a paracellular of transcellular chloride conductance pathway. This is mimicked by thapsigargin and ionomycin, which increase the level of intracellular calcium by promoting Ca^{2+} release for intracellular stores and the influx of Ca^{2+} from the bathing fluid, respectively. The ability of R-LK-CHO to block the activity of these pharmacological probes shows it cannot be an antagonist of the kinin receptor, but must act downstream of the second messenger pathway.

At present, we do not know the cellular action(s) of R-LK-CHO on Malpighian tubules that leads to it preventing stimulation of fluid secretion by Musdo-K, thapsigargin and ionomycin. It is clearly not a generalized toxic effect, because the same analog has diuretic activity in the cricket Malpighian tubule assay. Moreover, closely related aldehyde analogs tested on housefly tubules either have no activity (V-LK-CHO and Fmoc-RFF[Aib]WG-H) or stimulate fluid secretion (Fmoc-RAHPWG-H). R-LK-CHO may bind the kinin receptor on housefly tubules and become internalized, thereby gaining access to intracellular processes that couple a rise in intracellular calcium levels to the opening of the chloride conductance pathway.

Compounds in the hemolymph, including pesticide toxins, are actively transported into the lumen of the Malpighian tubules, and their rate of elimination is dependent on the rate of fluid secretion [10,21]. At high rates of excretion, the toxins do not reach the high concentrations that would allow them to diffuse back into the hemolymph down a concentration gradient. An agent capable of selective depression of fluid

secretion would be expected to allow pesticides to achieve higher concentrations in the hemolymph; and, in turn, likely reduce the amount of toxin required to kill an insect. Whatever the mode of action, the selective activity of R-LK-CHO on housefly tubules represents an important milestone and lead in our long-term goal of the development of environmentally-friendly insect management agents based on the insect kinins.

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